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Lymphocyte-Stromal Cell Interaction Induces IL-7 Expression by Interferon Regulatory Factors

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Key words; cytokine, cell adhesion, transcription factor, interferon, promoter, IL-7, stromal cell

Abstract

The interaction between lymphocytes and stromal cells plays important roles in coordinated development of early lymphocytes. IL-7 is an essential cytokine for early lymphocyte development produced by stromal cells in the thymus and bone marrow. Although IL-7 is induced by interaction of early lymphocytes and stromal cells, its molecular basis is still unknown. To address this question, we employed co-culture system with an IL-7-dependent pre-B cell line, DW34, and a thymic stromal cell line, TSt-4. Co-culture with DW34 cells enhanced the levels of IL-7 transcripts in TSt-4 cells. Interestingly, the co-culture also induced transcripts of IFN- α and IFN- β but not of IFN- γ . In addition, exogenous IFN- β stimulation increased the levels of IL-7 transcripts in TSt-4 cells. Next, to elucidate the molecular mechanism of IL-7 induction, we analyzed the IL-7 promoter activity by reporter assay. The IL-7 promoter showed specific transcriptional activity in TSt-4 cells. An interferon-stimulated response element (ISRE) in the IL-7 promoter was essential for the induction of IL-7 transcription by both co-culture and IFN- β stimulation. Finally, overexpression of wild-type and dominant-negative forms of interferon regulatory factors (IRFs) activated and repressed, respectively, the IL-7 promoter in TSt-4 cells. Collectively, these results suggested that IRFs activated by lymphocyte adhesion induce IL-7 transcription through ISRE in stromal cells and that type I IFNs may be involved in the activation of IRFs. Thus, this study implied a physiological function of the IFN/IRF signal during lymphocyte development.

1. Introduction

The cell interaction between lymphocytes and stromal cells plays a critical role in development and maintenance of the immune system. Bone marrow contains several types of mesenchymal stromal cells, which support the expansion of early B cells (Whitlock et al., 1985) and the homing and survival of memory CD4 T cells, plasma cells, and plasmacytoid dendritic cells (Kohara et al., 2007; Tokoyoda et al., 2004; Tokoyoda et al., 2009). Bone marrow stromal cells produce growth factors, cytokines, and chemokines such as SCF, IL-7, and CXCL12 (Tokoyoda et al., 2010). Early B cells adhere to stromal cells by interaction between two adhesion molecules, VLA-4 and VCAM-1, and receive growth and survival signals of these factors. Remarkably, the cell interaction stimulates the stromal cells to produce higher levels of IL-7 (Sudo et al., 1989). On the other hand, thymic microenvironment is mainly composed of epithelial and mesenchymal cells, both of which express SCF and IL-7 for T cell proliferation and survival (Rodewald et al., 1995; von Freeden-Jeffry et al., 1995). Thymic epithelial cells also produce a Notch ligand, Dll-4, for T cell lineage commitment (Hozumi et al., 2008; Koch et al., 2008). Moreover, they express MHC class I and class II molecules for positive and negative selection (Starr et al., 2003) and autoimmune regulator for self-tolerance (Anderson et al., 2002). In contrast, mesenchymal stromal cells in the thymus are important for differentiation and proliferation of epithelial cells in thymic organogenesis (Itoi et al., 2007). Interestingly, CD4⁺CD8⁻ or CD4⁻CD8⁺ single positive thymocytes are responsible for the maintenance of medullary thymic epithelial cells (Akiyama et al., 2008; Hikosaka et al., 2008). Therefore, not only lymphocytes depend on intact microenvironments for development, but microenvironments themselves depend on lymphoid cells to differentiate and maintain their integrity.

IL-7 is an essential cytokine for development and maintenance of early and mature

lymphocytes, produced by mesenchymal stromal cells and epithelial cells in bone marrow, thymus, lymph nodes, skin, and intestines. During early development, IL-7 transmits two kinds of signal in lymphocyte progenitors of bone marrow and thymus. One is for cell survival and proliferation, and the other is for V(D)J recombination in the IgH and TCR γ loci (Corcoran et al., 1998; Maki et al., 1996). In addition, IL-7 stimulates differentiation of post-selected CD8 T cells in the thymus by inducing Runx3 transcription factor (Park et al., 2010). In periphery, IL-7 is produced by fibroblastic reticular cells in T cell zones of lymph nodes and plays an important role in survival and homeostatic proliferation of naive and memory T cells (Link et al., 2007). Furthermore, IL-7 is induced in intestinal epithelial cells by IFN- γ and deteriorates inflammatory bowel diseases (Oshima et al., 2004; Totsuka et al., 2007).

The mechanism underlying IL-7 expression is only partially understood. It was reported that the transcription of IL-7 gene starts from two distinct regions: one from 480~640 bp upstream and the other from 200~220 bp upstream of the coding sequence (Ariizumi et al., 1995; Lupton et al., 1990). IL-7 transcripts were induced in bone marrow stromal cells by co-culture with pre-B cells as well as by stimulation with IL-1 (Sudo et al., 1989). Furthermore, IFN- γ treatment induces IL-7 transcription in keratinocytes and intestinal epithelial cells (Ariizumi et al., 1995; Oshima et al., 2004). The IL-7 promoter contains an interferon-stimulated response element (ISRE) at 270 bp upstream of the coding sequence. The ISRE is important for the transcription mediated by IFN- γ , and interferon regulatory factor (IRF) 1 and IRF2 activate the IL-7 promoter via the ISRE (Ariizumi et al., 1995; Oshima et al., 2004). Therefore, IFN- γ and IRFs play a crucial role in IL-7 induction during inflammation. However, the molecular basis of IL-7 induction by lymphocyte-stromal cell interaction is still unknown.

To define molecular mechanisms enhancing IL-7 transcription by cell interactions,

we employed a co-culture system with mesenchymal stromal cells and pre-B cells. The co-culture induced expression of IFN- α and IFN- β in the stromal cells. The IFNs then activated IRFs, which transactivated the IL-7 promoter via the ISRE. Thus, this study suggests that direct cell interaction between lymphocytes and stromal cells induces IL-7 transcription through type I IFNs and IRFs, implying a physiological function of the IFN/IRF signal in lymphocyte development.

2. Materials and methods

2.1. Cells

A thymic mesenchymal stromal cell line, TSt-4 (Watanabe et al., 1992), is maintained in RPMI-1640 medium containing 5% FBS, 50 μ M 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. A stromal cell-dependent pre-B cell line, DW34 (Nishikawa et al., 1988), is cultured on TSt-4 cell layer with the same medium. For co-culture, DW34 cells (4×10^6) were seeded on TSt-4 cell layer in T75 flask. After 5 days, DW34 cells were separated from TSt-4 cell layer by gentle pipetting. TSt-4 cells were then harvested by trypsin treatment. TSt-4 cells were stimulated with 1×10^3 U/ml recombinant mouse IFN- β (rMuIFN- β , TORAY Industries, Inc., Tokyo, Japan) or recombinant mouse IFN- γ (R&D Systems, Minneapolis, MN, USA) for 12 h. For Transwell culture, DW34 cells were seeded at 3.2×10^6 cells per well on the Transwell membrane with 0.4 μ m pore size (Corning Inc., Lowell, MA, USA). The Transwell membrane inserts were transferred into 6-well culture plates with pre-established TSt-4 cell layer. After 24 h, TSt-4 cells were harvested.

2.2. Plasmids

The mouse IL-7 promoter fragment from -460 to -126 bp of transcription initiation site was amplified from mouse genomic DNA by PCR and subcloned into pGL4.10 reporter vector (Promega, Madison, WI, USA) (p-460Luc). Sequences of the primers for the cloning of the IL-7 promoter are as follows: 5'-GACTCGAGGTTGCCAGAGAGGTTAGAAG-3' and 5'-CGAAGCTTCAGTTCAGGCAGCTGACTC-3'. The underlined nucleotides are XhoI

and HindIII restriction enzyme sites used for subcloning. p-460ISREmutLuc was obtained from p-460Luc by site-directed mutagenesis with the following oligonucleotide:

5'-ACCCAGCGCAAAGTAGAGGGCTGAGGGTACCCTGCTTAG-3' (the mutated nucleotides are underlined).

Mouse IRF1, IRF2, IRF3, IRF7, and IRF9 cDNAs were subcloned by RT-PCR into pCAGGS expression vector. For dominant negative (DN) form of IRF2, the portion from amino acid residues 155 through 349 was subcloned by PCR into pCAGGS vector. To construct pCAGGS-DN-IRF9, the XbaI-HindIII fragment of pEFΔp48 (Yoneyama et al., 1996) containing the DN-IRF9 cDNA was blunted and subcloned into pCAGGS vector.

IRF1, IRF2 and IRF9 short hairpin RNA (shRNA) plasmids were constructed by subcloning annealed oligonucleotides into the BamHI and EcoRI sites of pSIREN-RetroQ vector (Clontech, Mountain View, CA, USA), according to the manufacturer's instructions. Oligonucleotide sequences are as follows: IRF1, 5'-GAAGATAGCCGAAGACCTTAT-3'; IRF2, 5'-GCATCAACCAGGAATAGATAA-3'; IRF9, 5'-GTGTGTGGAGCTCTTCAAGAC-3'

2.3. Antibodies and cytokines

Anti-IRF2 and anti-IRF9 antisera were reported before (Yoneyama et al., 1996). Anti-IRF1, anti-IRF2 and anti-IRF9 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.4. RNA isolation and reverse transcription

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and

treated with RQ1 RNase-free DNase (Promega) to remove genomic DNA. Two microgram of total RNA was reverse-transcribed with ReverTra Ace (TOYOBO, Osaka, Japan) and random primer (Invitrogen).

2.5. PCR and real-time PCR

PCR was performed to amplify IL-7, upstream and downstream IL-7 promoters, IFN- α , IFN- β , IFN- γ , IRF1, IRF2, IRF3, IRF7, IRF9, toll-like receptor (TLR) 4, and receptor activator of nuclear factor- κ B (RANK) for 35 cycles at 95°C for 30 sec, 55-60°C for 30 sec, and 72°C for 1 min. Real-time PCR was performed with QuantiTect SYBR Green PCR Kit (QIAGEN, Hilden, Germany) for IL-7, IL-7 promoters, IFN- α , IFN- β , IRF1, IRF2, IRF3, IRF7 and IRF9, SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Japan) for IL-6, or Premix EX Taq (Takara Bio Inc.) for rRNA as internal control in triplicate by ABI 7500 Sequence detector (Applied Biosystems, Carlsbad, CA, USA). Primer sequences are shown in Table 1.

2.6. Reporter assay

TSt-4 cells were plated at 2×10^4 cells per well in 24-well plate and incubated overnight. After 24 h, the cells were transfected with each reporter plasmid containing firefly luciferase cDNA, pGL4.74 internal control reporter plasmid containing Renilla luciferase cDNA (Promega), and expression vectors using FuGENE 6 transfection reagent (Promega). After 12 h, TSt-4 cells were co-cultured with 4.5×10^5 cells per well of DW34 cells for 24 h or stimulated with 1×10^3 U/ml IFN- β for 12 h. Luciferase activity was measured with Dual-Luciferase reporter assay system (Promega) by a luminometer (Tropix TR-717 Microplate Luminometer; Applied Biosystems).

2.6. *shRNA*

PLAT-E cells were transfected with pSIREN-RetroQ vector containing IRF1 shRNA, IRF2 shRNA and IRF9 shRNA using FuGENE 6 transfection reagent. After 24 h, culture medium was replaced, and the culture supernatants were harvested after additional 24 h. TSt-4 cells were plated at 1×10^5 cells per 60 mm dish and infected with each retrovirus in the presence of 8 $\mu\text{g/ml}$ polybrene (Sigma-Aldrich, St. Louis, MO, USA) after 24 h. Stably transfected TSt-4 cells were selected in the medium containing 3 $\mu\text{g/ml}$ puromycin (Sigma-Aldrich). The purity ($> 95\%$) was confirmed by FACSCalibur (Becton Dickinson, Franklin Lakes, NJ).

3. Results

3.1. *IL-7 is induced by co-culture*

The induction of IL-7 was previously reported in a co-culture system with a bone marrow-derived stromal cell line, ST2, and a pre-B cell line, DW34 (Sudo et al., 1989). To investigate the molecular mechanism of IL-7 induction from stromal cells, we first checked the combination of stromal cells and lymphoid cells. We tested ST2 and TSt-4 (a thymic mesenchymal stromal line) as stromal cells, and DW34, preBRI (an IL-7-dependent pre-B cell line), and Jurkat lines as lymphoid cells. We found that the combination of TSt-4 and DW34 cells gave the strongest induction of IL-7 (data not shown). Thus, we cultured DW34 cells on TSt-4 cells. After 5 days, TSt-4 cells were separated from DW34 cells by gentle pipetting, and the levels of IL-7 transcripts were measured by real-time RT-PCR. While TSt-4 cells showed certain levels of IL-7 transcripts when cultured alone, co-culture with DW34 cells resulted in 4-fold increase of IL-7 transcripts in TSt-4 cells (Fig. 1A). In contrast, DW34 cells themselves did not produce IL-7 mRNA in the same culture (Fig. 1B). These results suggested that the co-culture with DW34 cells enhances the transcription of IL-7 gene in thymic stromal TSt-4 cells.

3.2. *Direct cell interaction is crucial for IL-7 induction*

To test whether direct cell interaction is necessary for IL-7 induction in co-culture, we used the Transwell culture system. TSt-4 cells were cultured with DW34 cells separated by Transwell membranes, and the levels of IL-7 transcripts were measured by real-time RT-PCR. The levels of IL-7 transcripts in the TSt-4 cells in Transwell culture were similar

with those in TSt-4 cells left alone (Fig. 1C). In contrast, TSt-4 cells directly co-cultured with DW34 cells showed significantly enhanced levels of IL-7 transcripts. These results indicated that the direct cell interaction with DW34 cells is required for efficient induction of IL-7 transcription in TSt-4 cells.

3.3. Transcription of the mouse IL-7 gene is initiated from two distinct sites

Two major IL-7 mRNA species have been detected in various tissues, including thymus (Namen et al., 1988). The different mRNAs were transcribed from alternative transcription initiation regions (Ariizumi et al., 1995; Oshima et al., 2004) (Fig. 2A). To examine whether the two regions are differentially regulated, we measured the levels of IL-7 transcripts by real-time RT-PCR with two primer pairs; the upstream pair amplifying the transcripts only from upstream initiation sites, and the downstream pair amplifying the transcripts from both upstream and downstream sites (Fig. 2B). The net downstream transcripts were calculated by subtracting upstream transcripts from downstream transcripts. The levels of downstream transcripts were over 100 times higher than those of upstream transcripts in both basal and inducible transcription of the IL-7 promoter (Fig. 2C and D). These results demonstrated that the transcription of the IL-7 promoter is mainly started from the downstream initiation sites and that the co-culture with DW34 cells enhances the IL-7 transcription from both the upstream and downstream sites in TSt-4 cells.

3.4. ISRE is essential for both basal and inducible transcription of the IL-7 promoter

As shown in Fig. 2, the transcripts from the downstream transcription initiation sites were dominant in both basal and inducible transcription of the IL-7 promoter. We next

characterized *cis* control elements in the mouse IL-7 promoter by reporter assay. A 460-bp fragment of the IL-7 promoter was joined to firefly luciferase reporter cDNA (p-460Luc) (Fig. 3A). TSt-4 cells were transiently transfected with the reporter vector and co-cultured with DW34 cells. The cells were recovered, and the promoter activity was analyzed by reporter assay. The wild-type IL-7 promoter showed a significant increase of transcriptional activity in TSt-4 cells both left alone and co-cultured as compared to the pGL4.10 control reporter vector (Fig. 3B). Previous reports demonstrated that the ISRE in the IL-7 promoter is important for IL-7 transcription in keratinocytes and epithelial cells (Ariizumi et al., 1995; Oshima et al., 2004). To test whether the ISRE is also essential in stromal cells, we constructed the p-460Luc reporter vector with a 4-bp mutation in the ISRE (p-460ISREmutLuc) (Fig. 3A). The p-460ISREmutLuc reporter showed a marked decrease of the promoter activity in both basal and inducible transcription (Fig. 3B). These results suggested that the co-culture with DW34 cells enhances the IL-7 promoter activity and that the ISRE plays a critical role in IL-7 transcription in TSt-4 cells.

3.5. Co-culture induces expression of type I IFNs and IRFs

Since the ISRE is essential for IL-7 transcription, we next searched for the transcription factors that mediate the IL-7 transcription via the ISRE. It is known that IRFs bind to the ISRE in IFN signaling. To test whether IRFs are induced in co-culture, transcript levels of the IRFs were measured by real-time RT-PCR in TSt-4 cells cultured alone or with DW34 cells. The levels of IFN- α and IFN- β transcripts were significantly increased in co-culture (Fig. 4A and B). On the other hand, IFN- γ mRNA was not detected in either culture (Fig. 4C). In parallel with the result of IL-7 transcription in the Transwell culture (Fig. 1C), direct cell interaction was also crucial for the induction of IFN- β (Fig. 4D). The levels of

IRF1, IRF2, IRF7 and IRF9 transcripts were considerably increased in co-culture compared with TSt-4 alone (Fig. 5), suggesting the involvement of IFN signaling pathway in the IL-7 transcription.

3.6. IFN- β induces the IL-7 gene expression

Because IFN- α and IFN- β transcripts were induced in co-culture, we next addressed the question whether IFN- α or IFN- β induces the transcription of IL-7 gene. We stimulated TSt-4 cells with recombinant IFN- β for 12 h and measured the levels of IL-7 transcripts by real-time RT-PCR. The IFN- β -treated TSt-4 cells showed higher levels of IL-7 transcripts than non-treated cells (Fig. 6A). The result suggested that IFN- β has potential for the induction of IL-7 transcription. Moreover, IFN- β stimulation enhanced the transcript levels of IRF1, IRF2, IRF7 and IRF9 but not IRF3 in TSt-4 cells (Fig. 6B-F). In addition, the activity of the IL-7 promoter was increased in response to IFN- β , while the promoter with mutant ISRE showed dramatically decreased activity (Fig. 6G). Although IFN- γ was undetected in co-culture, we treated TSt-4 cells with recombinant IFN- γ for 12 h. Unlike IFN- β , exogenous IFN- γ did not induce IL-7 expression (Fig. 6H). Taken together, these results suggested that the IL-7 transcription might be induced by IFN- β via ISRE in co-culture.

3.7. IRFs induce the transcription of the IL-7 promoter in co-culture

IFN signaling activates IRFs, which induce the transcription of inflammatory genes. To test whether IRFs activate the IL-7 promoter, TSt-4 cells were transfected with the p-460Luc reporter vector and the expression vector for IRF1, IRF2, IRF3, IRF7, or IRF9. Interestingly, exogenous IRF expression enhanced the IL-7 promoter activity except for IRF9

(Fig. 7A). In contrast, the reporter vector with mutant ISRE (p-460ISREmutLuc) was not affected by exogenous IRF expression, indicating that IRFs exert their effects via the ISRE in the IL-7 promoter. To determine the functional effect of endogenous IRFs in the IL-7 expression, we introduced DN forms of IRF2 (DN-IRF2) or IRF9 (DN-IRF9), or shRNAs specifically targeted to IRF1 or IRF2 into TSt-4 cells. The introduction of DN-IRF2 or DN-IRF9 inhibited the transcriptional activity (Fig. 7B). Moreover, the downregulation of IRF1 or IRF2, but not IRF9, by short hairpin RNAs significantly reduced the expression of IL-7 in TSt-4 cells (Fig. 7C). These results suggested that IRFs are involved in the induction of IL-7 transcription by IFN- β .

4. Discussion

In this study, we employed the co-culture system with the thymic stromal cell line, TSt-4, and the pre-B cell line, DW34, to analyze the molecular mechanism of IL-7 production from stromal cells. We first showed that the co-culture with DW34 cells induces IL-7 transcription in TSt-4 cells (Figs. 1 and 2). Direct cell interaction is crucial for the induction. We also demonstrated that the ISRE in the IL-7 promoter is essential for basal and inducible transcription (Fig. 3). In addition, the co-culture induces the expression of type I IFNs and IRFs (Figs. 4 and 5), and IFN- β induces the IL-7 transcription (Fig. 6). We finally indicated by reporter assay that IRFs are involved in the IL-7 induction by co-culture (Fig. 7). These results collectively suggested that the direct cell interaction with lymphocytes induces IL-7 transcription in stromal cells by IFNs and IRFs.

The transcription of IL-7 gene starts from two different regions in the promoter (Ariizumi et al., 1995; Oshima et al., 2004). However, it has been unclear whether the upstream and downstream transcription initiation regions are selectively controlled or equally used in stromal cells. We differentially quantitated the levels of IL-7 transcripts started from upstream and downstream regions. The levels of the transcripts from the downstream region are over 100 times higher than those from upstream region in both basal and inducible transcription (Fig. 2C and D). Thus, our study is the first report showing that the downstream transcription initiation sites are mainly used in the IL-7 promoter in stromal cells.

IFNs and IRFs play an essential role in transcription of IL-7 gene. It has been reported that IL-7 transcription is induced in keratinocytes by IFN- γ (type II IFN) via the ISRE in the IL-7 promoter (Ariizumi et al., 1995) and that IRF1 and IRF2 are involved in IL-7 transcription in intestinal epithelial cells (Oshima et al., 2004). Although the ISRE is essential for the IL-7 transcription in stromal cells (Fig. 3B), IFN- γ was not detected in

co-cultured TSt-4 cells (Fig. 4C). Interestingly, the levels of IFN- α and IFN- β transcripts were significantly increased in TSt-4 cells co-cultured with DW34 cells compared with TSt-4 cells left alone (Fig. 4A and B). In addition, IFN- β induces IL-7 transcription via the ISRE in the IL-7 promoter (Fig. 6G). IFN- α and IFN- β transmit signals through a shared receptor complex, IFNAR, whereas IFN- γ is recognized by another receptor, IFNGR. Ligand binding with IFNAR results in the formation of an IRF signaling complex and the activation of the ISRE (Honda et al., 2006). Therefore, our study suggested that IFNs and IRFs are involved in the transcription of IL-7 gene in stromal cells.

TSt-4 cells expressed basal levels of IL-7 and IFN- α/β even without DW34 cells (Figs. 1 and 4). A recent study demonstrated that fibroblastic reticular cells, key stromal cells in lymph nodes that produce IL-7, upregulate transcripts for both IL-7 and IRF7 12 hours after in vivo LPS stimulation (Malhotra et al., 2012). It might be possible that trace amounts of endotoxin in the culture might have induced IFN- α/β and then IL-7.

There might be different mechanisms to induce IL-7 in co-culture. First, it is possible that IFN- α and IFN- β are involved in induction of IL-7. Second, IL-6 is transiently induced in co-culture and has potential to induce IL-7 (data not shown). IL-6/STAT3 signaling may regulate IL-7 transcription in stromal cells. In fact, several STAT3 binding sites exist in the IL-7 promoter (data not shown). However, we failed to detect the IL-6-dependent activity of IL-7 transcription with the p-460Luc reporter vector that contains a STAT3 binding site near the ISRE (data not shown). It might be that upstream STAT3 binding sites not included in the p-460Luc vector are necessary for IL-6/STAT3-dependent transcription of the IL-7 gene.

The mechanism how direct cell interaction with lymphocytes induces type I IFN production in stromal cells has not been determined yet. Because cell interaction is crucial for the induction of IL-7 and IFN- β , some adhesion molecules might trigger the IFN- β

production. We have tested expression of several candidate molecules for direct cell interaction in TSt-4 cells by RT-PCR. We observed the expression of VCAM-I but not RANK, which transmits signals via NF- κ B (data not shown). In addition, we detected the expression of TLR4 that activates IRF3 and induces IFN- β transcription (data not shown). Further studies will be required to elucidate the mechanism.

While IRF9 expression and shRNA failed to affect the transcriptional activity of the IL-7 promoter, DN-IRF9 expression reduced it (Fig. 7). We don't have any convincing results to explain the contradictory results. IRF1 and IRF2 probably play a major role in transcription of IL-7 gene, as indicated by knockdown experiments (Fig. 7C). Therefore, it is reasonable that IRF9 shRNA showed only minimal effects. In addition, because IRF9 functions by forming ISGF3 complex with STAT1 and STAT2, the expression of IRF9 alone (which might be at lower levels) might not be enough to increase luciferase activities. In contrast, the overexpression of DN-IRF9 (which might be at higher levels) might have some effects such as binding to and sequestering transcriptional coactivators, which resulted in reducing the transcriptional activity.

IFNs may play an essential role in physiological production of IL-7 in the thymus and bone marrow. Although our study is conducted by in vitro co-culture system, it is possible that the results reflect lymphocyte-stromal cell interactions in vivo. This study suggested that direct lymphocyte-stromal cell interaction induces type I IFNs, resulting in mobilization of IRFs and activation of the IL-7 promoter. Therefore, it implied that type I IFNs might be involved in IL-7 production in vivo. In this scenario, the transcripts of IFN- α and IFN- β were detected in the thymus (data not shown). Our study will stimulate future search for physiological functions of type I IFNs during lymphocyte development.

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Abbreviations

DN	dominant negative
IRF	interferon regulatory factor
ISRE	interferon-stimulated response element
RANK	receptor activator of nuclear factor- κ B
shRNA	short hairpin RNA
TLR	toll-like receptor

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Figure Legends

Fig. 1. IL-7 is induced by direct lymphocyte-stromal cell interaction.

(A) TSt-4 cells either cultured alone or with DW34 cells were harvested after 5 days. The levels of IL-7 transcripts were measured by real-time RT-PCR. The levels of IL-7 transcripts were normalized with those of 18S rRNA. Values are the mean \pm S.E. of triplicate data points from a representative experiment. Data are representative of two independent experiments. (B) The IL-7 transcripts from TSt-4 and DW34 cells were amplified by RT-PCR and analyzed by 2% agarose gel electrophoresis. (C) TSt-4 cells were harvested after 24 h of Transwell culture. The levels of IL-7 transcripts were measured as in Fig. 1A. Data are representative of two independent experiments. The Student's *t* test for unpaired data was used to compare the values between two groups (**, $p < 0.01$; ***, $p < 0.001$; n.s., not significant).

Fig. 2. Transcription of mouse IL-7 promoter mainly starts from downstream initiation sites.

(A) Nucleotide sequence alignment of the human (above) and mouse (below) IL-7 promoters. Transcription initiation sites are shown by arrowheads (Oshima et al., 2004). The translation start site (ATG) is indicated in bold. The numbers represent the nucleotides from the translation start site. ISRE is boxed. (B) The regions amplified by upstream or downstream primer pairs are indicated. (C) and (D) TSt-4 cells were cultured with or without DW34 cells for 5 days. The levels of IL-7 transcripts were measured by real-time RT-PCR with upstream- and downstream-specific primer pairs as in Fig. 1A. The Student's *t* test for unpaired data was used to compare the values between two groups (***, $p < 0.001$).

Fig. 3. ISRE is essential for the transcription of the IL-7 promoter.

(A) Schematic illustration of luciferase reporter constructs. The mouse IL-7 promoter fragment from -460 to -126 bp with or without mutated ISRE was flanked by luciferase cDNA. (B) Transcriptional activation of the IL-7 promoter by co-culture. TSt-4 cells were transiently transfected with mixture of the pGL4.10 reporter plasmids and the *Renilla* luciferase control vector. After 12 h, TSt-4 cells were co-cultured with DW34 cells for 24 h. Firefly luciferase activity in the whole cell lysate was normalized by *Renilla* luciferase activity. The activity of pGL4.10 control plasmid was set to 1. Values are the mean \pm S.E. of triplicate data points from a representative experiment. Data are representative of three independent experiments. The Student's *t* test for unpaired data was used to compare the values between two groups (**, $p < 0.01$).

Fig. 4. Co-culture induces the expression of type I IFNs.

(A)-(C) TSt-4 cells were co-cultured with or without DW34 cells for 5 days. The levels of IFNs (IFN- α , IFN- β , and IFN- γ) transcripts were measured by real-time RT-PCR as in Fig. 1A or agarose gel electrophoresis. (D) TSt-4 cells were co-cultured with or without DW34 cells in Transwell culture system. The levels of IFN- β transcripts were measured by real-time RT-PCR. Values are the mean \pm S.E. of triplicate data points from a representative experiment. Data are representative of two independent experiments. The Student's *t* test for unpaired data was used to compare the values between two groups (*, $p < 0.05$; **, $p < 0.01$; n.s., not significant).

Fig. 5. Co-culture induces the expression of IRFs.

(A)-(E) TSt-4 cells were co-cultured with or without DW34 cells for 5 days. The levels of IRFs (IRF1, IRF2, IRF3, IRF7, and IRF9) transcripts were measured by real-time RT-PCR as in Fig. 1(A). Values are the mean \pm S.E. of triplicate data points from a representative

experiment. Data are representative of two independent experiments. The Student's *t* test for unpaired data was used to compare the values between two groups (*, $p < 0.05$; **, $p < 0.01$; n.s., not significant).

Fig. 6. IFN- β induces the transcription of IL-7 gene.

(A)-(F) TSt-4 cells were treated with 1×10^3 U/ml of recombinant mouse IFN- β or (H) 10 or 50 ng/ml of recombinant mouse IFN- γ for 12 h. The levels of IL-7 and IRFs transcripts were measured by real-time RT-PCR as in Fig. 1A. Values are the mean \pm S.E. of triplicate data points from a representative experiment. Data are representative of three independent experiments. The Student's *t* test for unpaired data was used to compare the values between two groups (*, $p < 0.05$). (G) Transcriptional activation of the IL-7 promoter by IFN- β . TSt-4 cells were transiently transfected with mixture of the pGL4.10 reporter plasmids and the *Renilla* luciferase control vector. After 12 h, TSt-4 cells were treated with 1×10^3 U/ml of recombinant mouse IFN- β for 12 h. Reporter assay was carried out as in Fig. 3B. The activity of pGL4.10 control plasmid was set to 1. Data are representative of three independent experiments. The Student's *t* test for unpaired data was used to compare the values between two groups (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n.s., not significant).

Fig. 7. IRFs are involved in IL-7 induction.

(A) TSt-4 cells were transiently transfected with mixture of the pGL4.10 reporter plasmids, IRF expression vectors, and the *Renilla* luciferase control vector. After 12 h, TSt-4 cells were harvested and subjected to reporter assay as in Fig. 3B. The promoter activity of pGL4.10 control plasmid in mock transfection was set to 1. Data are representative of two independent experiments. (B) TSt-4 cells were transiently transfected with the mixture of the pGL4.10 reporter plasmids, the expression vectors of DN forms of IRF2 or IRF9, and the *Renilla*

luciferase control vector. After 12 h, TSt-4 cells were co-cultured with DW34 cells for 24 h. Reporter assay was carried out as in Fig. 3B. The promoter activity of p-460Luc plasmid in mock transfection was set to 100. Data are representative of three independent experiments. The Student's *t* test for unpaired data was used to compare the values between two groups (*, $p < 0.05$). (C) TSt-4 cells were stably transfected with control, IRF1, IRF2 and IRF9 shRNA vectors. The levels of IL-7 transcripts were measured by real-time RT-PCR. Values are the mean \pm S.E. of triplicate data points from a representative experiment. Data are representative of two independent experiments. The Student's *t* test for unpaired data was used to compare the values between two groups (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Fig. 1

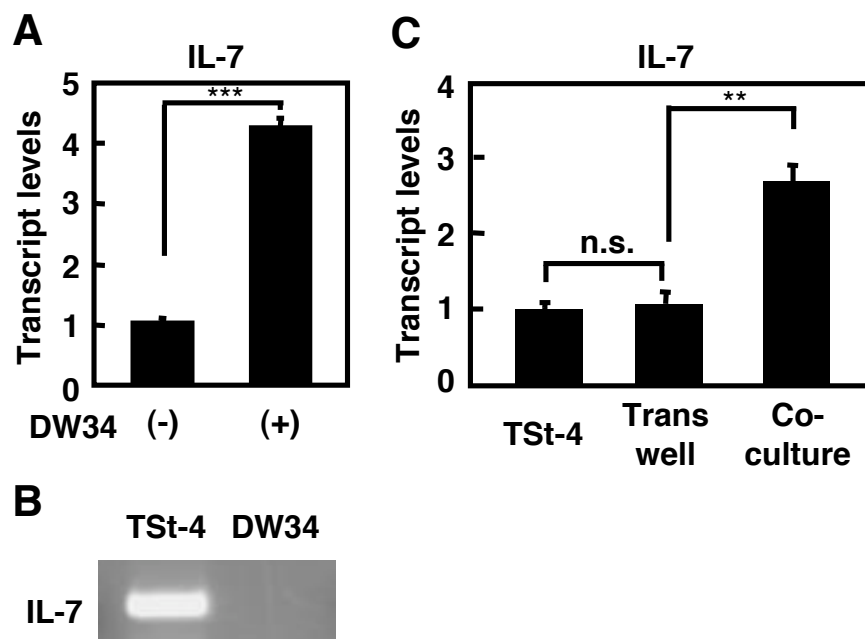


Fig. 2

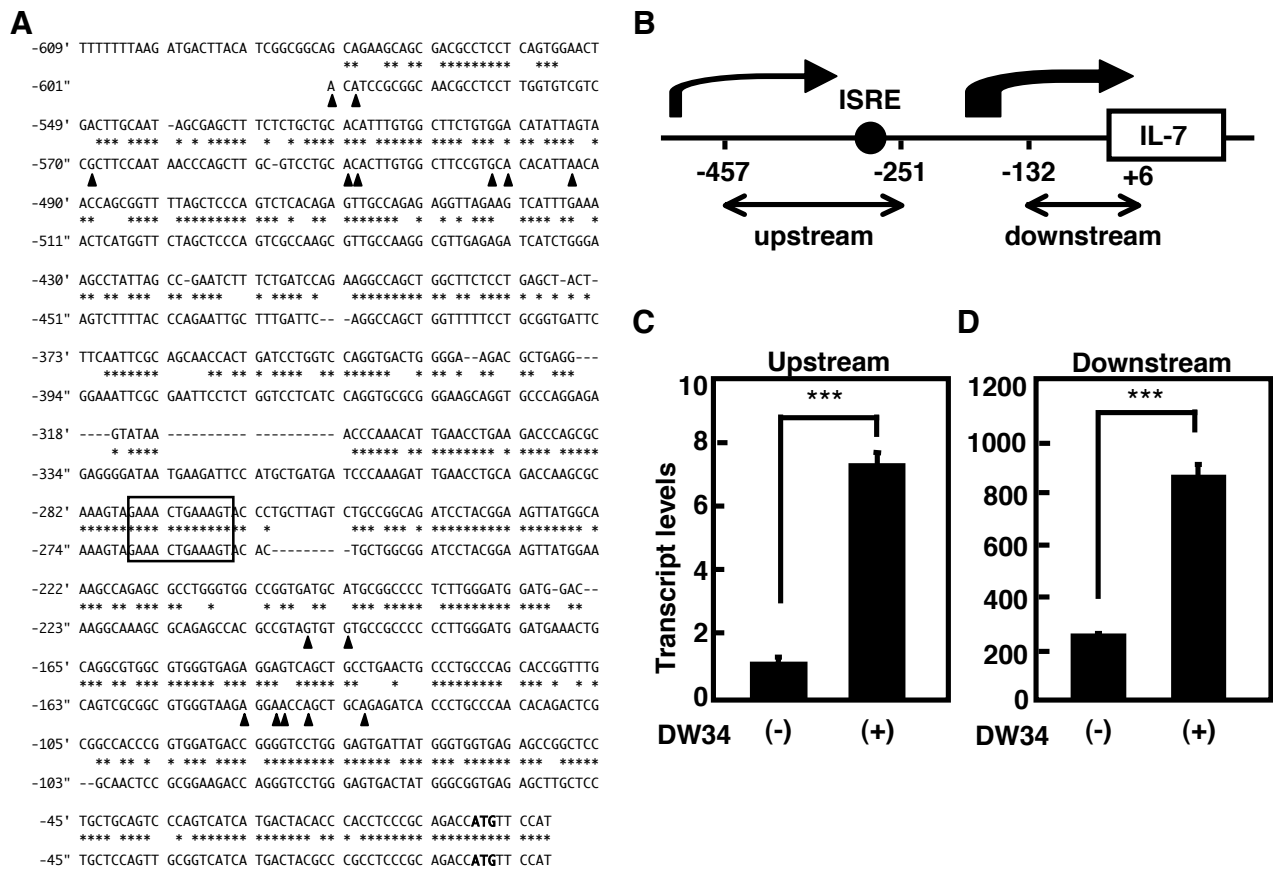


Fig. 3

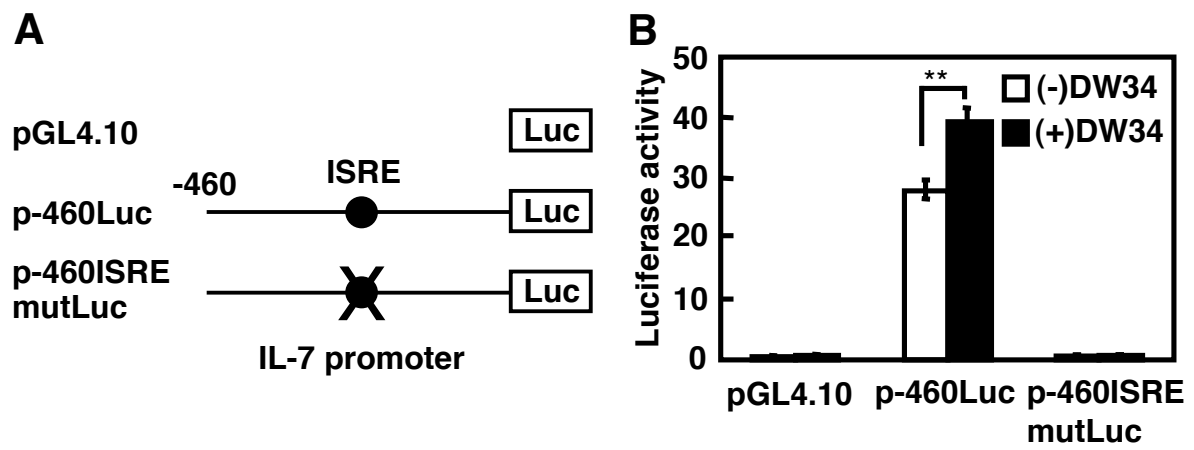


Fig. 4

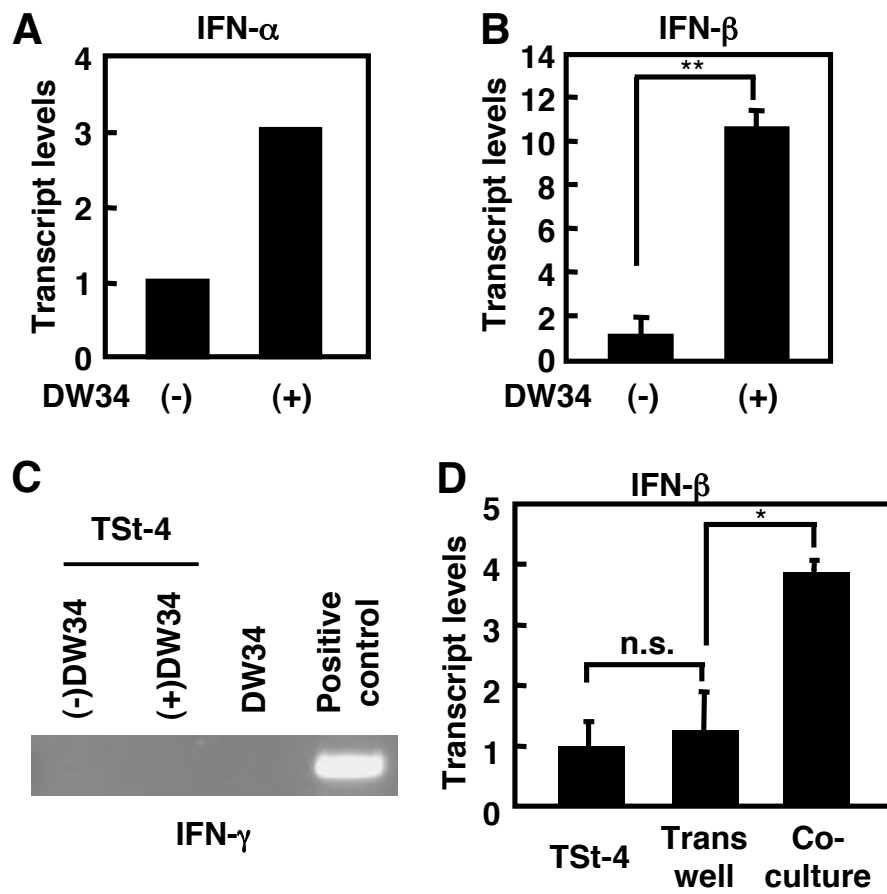


Fig. 5

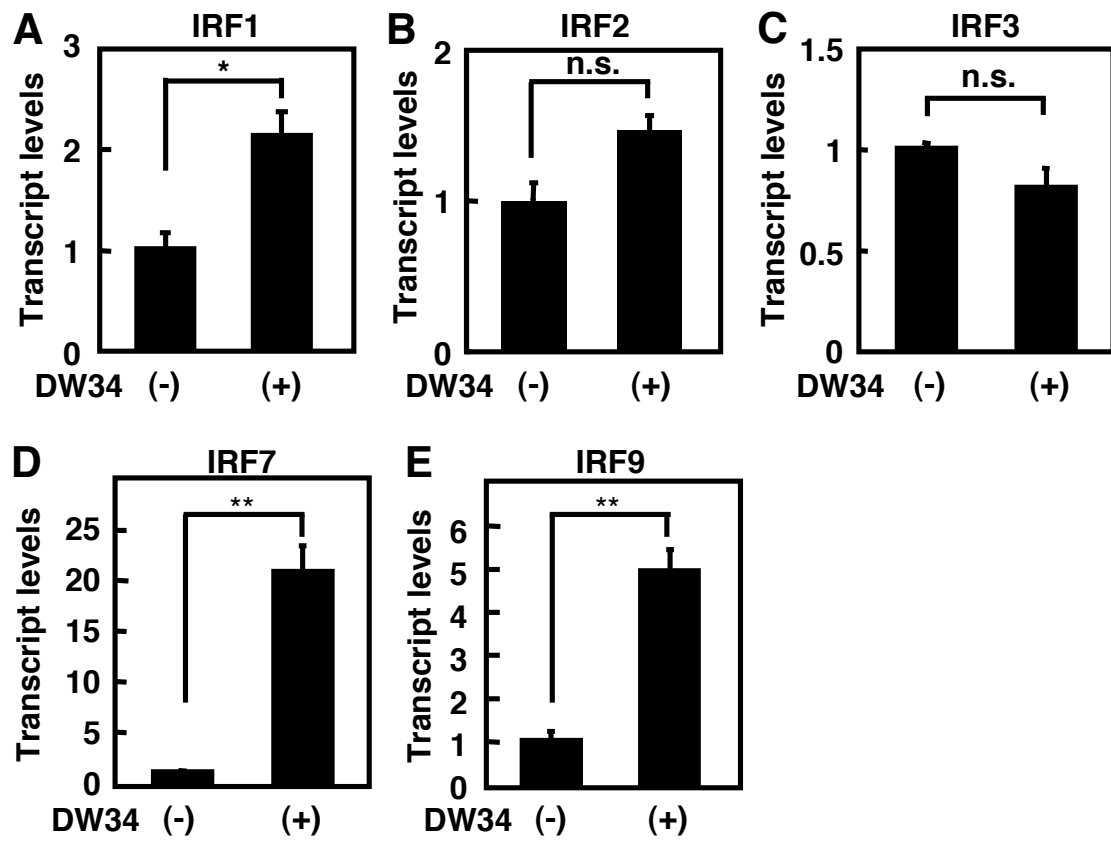


Fig. 6

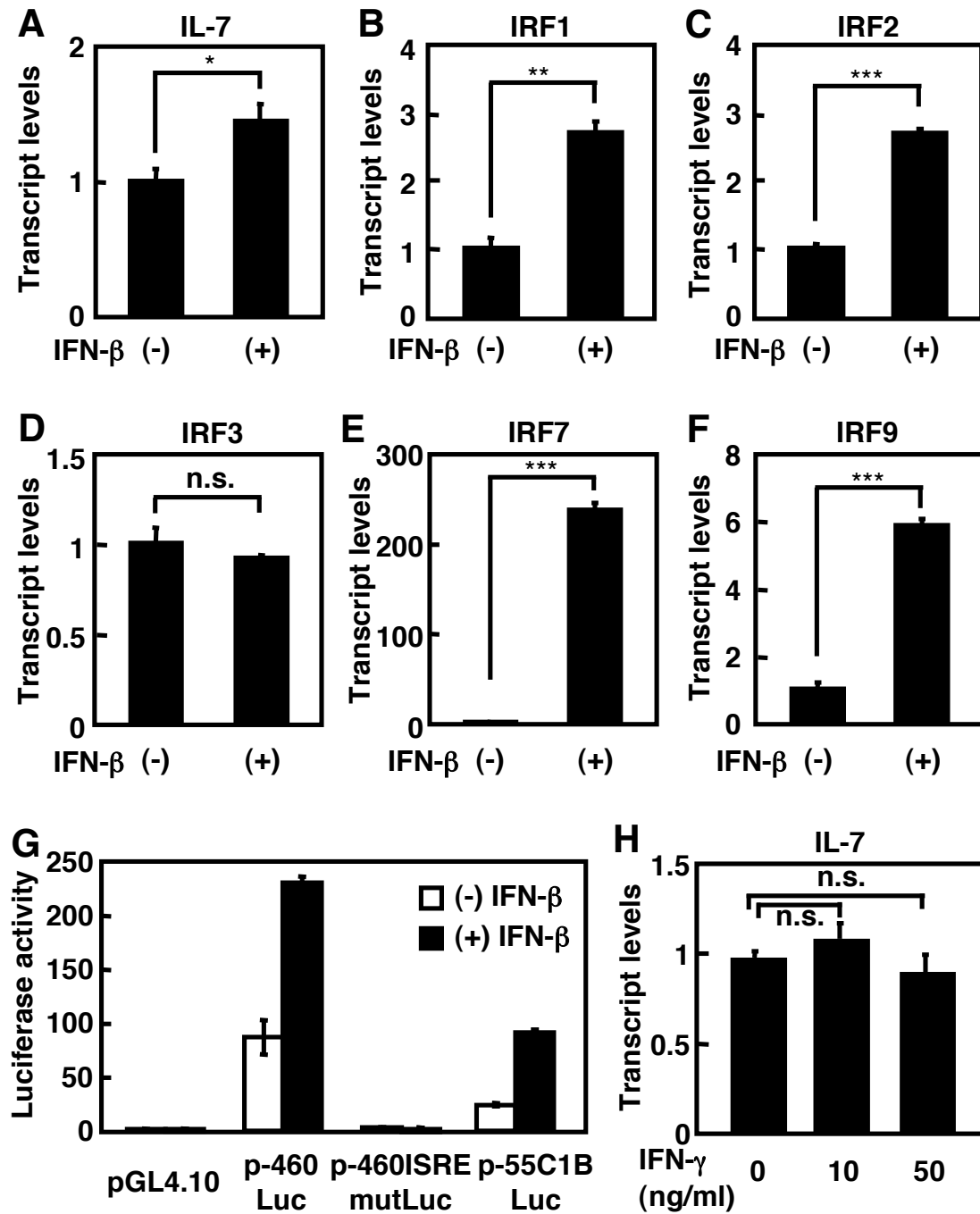


Fig. 7

